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GONADAL DEVELOPMENT DURING THE ANNUAL REPRODUCTIVE CYCLE OF *COMANTHUS JAPONICA* (ECHINODERMATA: CRINOIDEA)

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Echinoderm gonads have been described from periodic histological examinations made throughout the annual reproductive cycle in the following classes: Holothuroidea (Tanaka, 1958); Ophiuroidea (Patent, 1969; Fenaux, 1970, 1972); Asteroidea (Pearse, 1965; Chia, 1968; Crump, 1971); and Echinoidea (Yoshida, 1952; Fuji, 1960; Holland and Giese, 1965; Holland, 1967; Fenaux, 1968; Pearse and Phillips, 1968; Holland and Holland, 1969; Holland and Rommel, 1969; McPherson, 1969; Pearse, 1969a, 1969b, 1970; Gonor, 1973a, 1973b). No comparable study has yet been published for any species of the echinoderm class Crinoidea. Therefore, the present investigation describes gonadal development during the annual reproductive cycle of *Comanthus japonica* (Müller), an unstalked crinoid. The anatomy and histology of the crinoid reproductive system are diagrammed in Figure 3 of Holland (1975).

Materials and Methods

We studied a population of *Comanthus japonica* living on rocks at the entrance of Koaziro Bay, Kanagawa Prefecture, Japan. At each collection we captured 3 to 25 adult crinoids by diving in a few meters of water. The distribution of male, female and unsexable animals was as follows: 14 December 1972 (1 M + 7 F); 1 March 1973 (3 M + 7 F); 7 May 1973 (5 M + 4 F); 1 July 1973 (3 M + 4 F); 4 August 1973 (5 M + 4 F); 18 August 1973 (5 M + 4 F); 2 September 1973 (2 M + 3 F); 15 September 1973 (3 F); 24 September 1973 (8 F); 27 September 1973 (14 F); 30 September 1973 (12 F); 2 October 1973 (3 M + 13 F); 7 October 1973 (4 M + 14 F); 11 October 1973 (13 M + 12 F); 22 October 1973 (5 M + 4 F + 4 U); 8 November 1973 (4 M + 7 U); 24 November 1973 (2 F + 8 U); and 1 February 1974 (4 M + 6 F). For the samples of 15 September 1973 through 2 October 1973, we deliberately collected more females than males; however, for 14 December 1972 and 7 October 1973, the predominance of females over males was presumably due to random sampling error. The collected animals had 28 to 42 arms each and had disc diameters from 2.4 to 3.7 cm; crinoids in this size range had presumably been through at least one annual reproductive cycle before our study commenced.

On the day of collection, several genital pinnules were removed from each animal about a fifth of the way between the arm base and the arm tip; they were fixed at once in sea water-Bouin's fluid. The fixation of 2 October 1973 was

several hours before spawning took place. After fixation, genital pinnules were dehydrated in ethanol, cleared in xylene, embedded in paraffin and sectioned at 7 μ. Some sections were stained with alcian blue at pH 3, azure A, mercuric bromphenol blue or periodic acid-Schiff (PAS) under conditions stated on page 284 of Holland and Nimitz (1964); before staining with PAS, glycogen was removed with diastase (Pearse, 1960). Other sections were stained with haematoxylin and eosin, with haematoxylin and PAS, or with alcian blue and PAS.

For each gonad, the volumes of several cell populations were determined by a method not previously described. A histological cross section was cut a fourth of the way between the base and the tip of the gonad. The section was stained with haematoxylin and PAS, and its image was projected by means of a *camera lucida* onto a sheet of aluminium foil at a magnification of 88×. For each ovary, the outlines of the post-pachytene oocytes (without extracellular jelly, if present) and the lumen were outlined on the foil. Regions outlined for each testis were the spermatogonia, the spermatocytes together with spermatids and the spermatozoa. Each aluminium foil image was cut out and weighed to the nearest tenth of a milligram. These weights were then divided by the weight of an aluminium foil square 88 mm on a side in order to convert weight to area. The gonad (which actually tapers gently along the distal fourth of its length) was assumed to be a solid cylinder with a constant diameter all along its length. Gonad length (measured from dissections of fixed pinnules adjacent to those prepared for histology) was multiplied by area to yield the volume of each region in mm^3 per gonad.

Fluctuations in the size-frequency structure of the post-pachytene oocyte population were studied by the frequency polygon method of Pearse (1965, p. 53). In histological sections of each ovary, the diameters of the first fifty oocytes encountered in nucleolar section were measured with an occular micrometer. Since most oocytes were somewhat elliptical in outline, the diameter was calculated by adding the long and short axes of the ellipse and dividing by two. When an oocyte was dented (as in Fig. 15), the diameter was measured from the ridge crests on one side of the cell to the ridge bases on the opposite side of the cell. The size range from 0 to 250 μ was divided into twenty size classes at 12.5-μ intervals, and the percentage of oocytes in each size class was calculated.

Instantaneous relative growth rates were calculated for the post-pachytene oocytes according to Brody (1945, p. 508). The volume of the largest (and presumably first-produced) oocyte was calculated from the average frequency polygon for each collection; the volume was obtained from the diameter at the upper limit of the largest size class to contain at least 1% of the oocytes. On the assumption that the annual reproductive cycle follows essentially the same course year after year, we plotted the data point for 24 November 1973 as 24 November 1972 and the data point for 1 February 1974 as 1 February 1973. Volumes were plotted on a semilogaramithic scale against time in days after 15 November, the birth date of the first-produced oocyte, which was 8 μ in diameter at birth. Similarly, the volume of the smallest (and presumably last-produced) oocyte was calculated from the average frequency polygon for each collection; the volume was obtained from the diameter at the lower limit of the smallest size class to contain at least 1% of the oocytes. These volumes were plotted against time in days after 15 February 1973, the birth date of the last-produced oocyte, which was 8 μ in diameter at birth.

The total number of post-pachytene oocytes per ovary was calculated for females collected from March 1973 through October 1973. The total volume of all oocytes in the ovary (obtained by the method described three paragraphs above) was divided by the volume of an average oocyte; this latter quantity was obtained by calculating the volumes of the 50 oocytes used for the frequency polygon analysis and dividing their sum by 50. These calculated numbers of oocytes per ovary were compared with direct counts of the number of oocytes per ovary. Counts of all oocytes were made in a complete serial reconstruction of one ovary from a female collected on 1 March 1973. Also, counts of all oocytes were made by dissecting one ovary from each of five females collected on 2 October 1973. Both of these direct counting methods proved too time consuming for more extensive use.

The growth of the nucleus and nucleolus relative to oocyte size was determined from measurements of 100 post-pachytene oocytes that had been reconstructed serially. For each of the following collections, ten oocytes with the following ranges in diameter were measured: 14 December 1972 (8–40 μ), 1 March 1973 (30–80 μ), 7 May 1973 (45–100 μ), 1 July 1973 (80–150 μ), 2 September 1973 (120–170 μ), and 15 September 1973 (150–200 μ). For each of the following collections, twenty oocytes with the following ranges in diameter were measured: 24 September 1973 (158–213 μ, all with extracellular jelly and a smooth surface) and 2 October 1973 (157–216 μ, all with extracellular jelly and a dented surface).

The deposition of extracellular jelly around oocytes and the development of the dented oocyte surface were studied by plotting the frequency polygon for each female collected from 15 September through 2 October 1973. In these oocytes, it was noted whether annulate lamellae were present or absent; these organelles, which appear by light microscopy as basophilic inclusions in the oocyte cytoplasm, were actually identified as annulate lamellae by comparison of adjacent Epon thick sections with ultrathin sections examined by electron microscopy (Holland, unpublished).

Results

The first parts of this section deal with gross anatomy and tissues of the reproductive system; the spawning dates delimiting the annual reproductive cycle are also described. These topics, with their definitions, put the biometric results into context. The biometric results, in turn, provide a background for the cytological results.

Gross anatomy of C. japonica, *with special reference to the reproductive system*

The crinoid body consists of a central disc from which the arms radiate; each arm bears numerous side branches known as pinnules (Fig. 3 in Holland, 1975). For *C. japonica*, the 50 or so pinnules nearest the disc are called the genital pinnules, since each contains a gonad. Therefore, a large female with 40 arms would have about 2,000 ovaries, while a large male with 40 arms would have about 2,000 testes. All the gonads of a given animal are at virtually the same stage of development. Moreover, most of the gonads of a given animal are about the same size; only the most distally located gonads of each arm are markedly smaller than the other gonads (in the present study, these smaller gonads were never the ones

sampled). Each gonad, which is elongate and approximately cylindrical, varies greatly in size during the annual reproductive cycle. When smallest, in November, the width is about 0.05 mm, and the length is about 6 mm. When largest, in early October, the width is about 1.2 mm, and the length is about 8 mm.

The tissues of the gonads

Both the ovary and the testis are composed of three layers arranged concentrically around a central, blind lumen. The *outer layer* of the gonad consists mainly of a sheet of visceral peritoneal cells. Muscle cells and nerve cells, which are also present, are very small and can be demonstrated clearly only by electron microscopy (Holland, 1971 and unpublished observations). For our purposes, the outer layer needs no further biometric or cytological description.

The *inner layer* of the gonad is the germinal epithelium, and its structure depends both on the sex of the animal and on the time of year. The inner layer of the ovary is made up of some combination of non-germinal cells, oogonia, spireme oocytes and post-pachytene oocytes. The cytodifferentiative sequence of these female germinal cells is: oogonia, spireme oocytes and post-pachytene oocytes. The inner layer of the testis is made up of some combination of non-germinal cells, spermatogonia, spermatocytes and spermatids. Spermatozoa are not a part of the inner layer, being located instead in the testicular lumen. The cytodifferentiative sequence of the male germinal cells is: spermatogonia, spermatocytes, spermatids and spermatozoa. In the ovaries and testes, several of the germinal cell populations undergo conspicuous fluctuations in volume, which will be described in the biometric part of the results section. All of the non-germinal and germinal cell types of the inner layer and lumen will be described in the cytological part of the results section.

Interposed between the outer and inner layers of the gonad is the *intermediate layer*, consisting of an inconspicuous matrix of haemal fluid in which are embedded the gonadal accessory cells. The size of the accessory cell population depends on the time of year, both in the ovary and in the testis. When enlarged, this cell population presses into the center of the gonad; at such times, the gonadal lumen, although reduced to a mere slit, always remains bounded by the inner layer. Fluctuations in the volume of the accessory cell population and the structure of the individual accessory cells will not be described in the present investigation.

Spawning dates delimiting the 1972–73 reproductive cycle

The population of *C. japonica* living in Koaziro Bay typically spawns each year on a single afternoon in October (Dan and Kubota, 1960). However, the annual reproductive cycle of 1972–73 was not typical, since it began with spawning on two consecutive afternoons (11 and 12 October 1972) and ended with spawning on at least five afternoons in October 1973. We did not anticipate such a complicated spawning pattern, and thus our sampling procedure left much to be desired. During the first half of October 1973, we collected crinoids every day or two. Some animals collected on 2, 3, 4, 17 and 18 October spawned after being brought into the laboratory on the day of collection. Moreover, some animals were observed spawning in the field during the afternoons of 3, 4 and 18 October; no field ob-

servations were made on 2 October, and those made on 17 October demonstrated no spawning animals. Random samples of animals collected on 7 and 11 October 1973 revealed that the crinoids spawning on 2, and 3 and 4 October accounted for about 40% of the males and about 40% of the females. After the spawnings of 17 and 18 October, a random sample collected on 22 October revealed that the remainder of the females had spawned; however, a few unspawned (or perhaps partially spawned) males were still present. By 8 November, all the collected males had spawned. Thus the 1972–73 reproductive cycle, which began in the second week of October 1972, ended for about 40% of the population in the first week of October 1973 and probably ended for the remaining 60% of the population in the third week of October 1973.

Volume fluctuations in the oocyte population and ovarian lumen

Figure 1A shows fluctuations in the volume of the population of post-pachytene oocytes and in the volume of the ovarian lumen. The population of oocytes increased in volume throughout the winter, spring and summer. This increase resulted from the enlargement of the individual oocytes, even though the total number of cells was decreasing from mid-winter through spawning in October (as will be demonstrated below). At spawning, the population volume decreased suddenly to a very small value, which declined to zero in the month that followed. The ovarian lumen, present but immeasurably small for much of the year, became enlarged in summer and was presumably filled with fluid (although some of the enlargement may have been a shrinkage artifact caused by fixation). In some ovaries, the enlarged lumen contained portions of degenerating oocytes, as illustrated on plate XV, Figure 4 in Dan and Dan (1941a). In the days immediately after spawning in October, the ovarian lumen remained somewhat distended; however, by a month after spawning, the lumen had become too small to measure.

Volume fluctuations in male germinal cell populations

Figure 1B shows fluctuations in the volumes of the germinal cell populations of the testis. For each cell type, the volume of the cell population is proportional to the number of cells present, since the sizes of individual cells do not vary greatly during the year. The population volume of the spermatogonia increased during winter, spring and early summer. An analysis of this volume increase by the method of Brody (1945, p. 513) revealed that, during this part of the year, the spermatogonia had a maximum mean cell cycle time of 35 days (we assumed that the growth fraction was unity and that no cell death occurred). Subsequently, during the latter part of August and during September, the population volume of the spermatogonia decreased rapidly and finally declined zero in the month after spawning. The population volume of the spermatocytes together with spermatids increased from zero in early July to maximum values during August and September. At spawning in early October, the volume fell suddenly and then declined to zero in the following weeks. The population volume of the spermatozoa was zero in early July, increased markedly during late summer, and then decreased suddenly at spawning. Finally, in the weeks that followed spawning, the volume declined to zero.

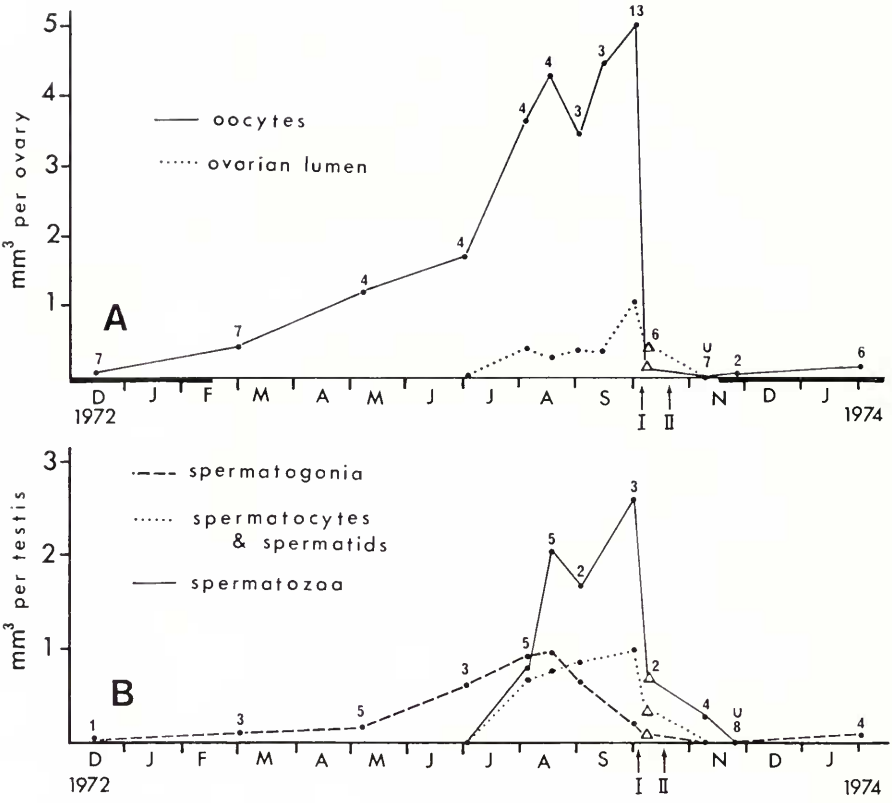

FIGURE 1. Volume fluctuations of gonadal regions during the annual reproductive cycle of *Comanthus japonica*. The number of individual animals averaged for each sample is shown above the date of collection. The spawnings of the first and third week of October 1973 are marked, respectively, by arrows I and II. For the triangular data points, only spawned animals in the collection were taken into account. For the data points labeled *U*, the animals were unsexable; (A) volume per ovary of the post-pachytene oocyte population and of the ovarian lumen. The thickened segments of the horizontal axis indicate the approximate interval during each annual cycle when precursors of the post-pachytene oocytes (namely, oogonia and spireme oocytes) were present in the ovary; (B) volume per testis of the male germinal cell populations.

Fluctuations in the size-frequency structure of the oocyte population

Changes in the size-frequency structure of the post-pachytene oocytes have been demonstrated by a frequency polygon analysis (Fig. 2). Each of the eleven average frequency polygons has been plotted against the date of collection. For most collections, the polygon of each individual female was approximately congruent with the average polygon; however, four of seven females collected on 1 March 1973 had distinctly bimodal frequency polygons. Otherwise, the individual polygons were unimodal, aside from relatively slight irregularities, which were presumably due to random sampling error.

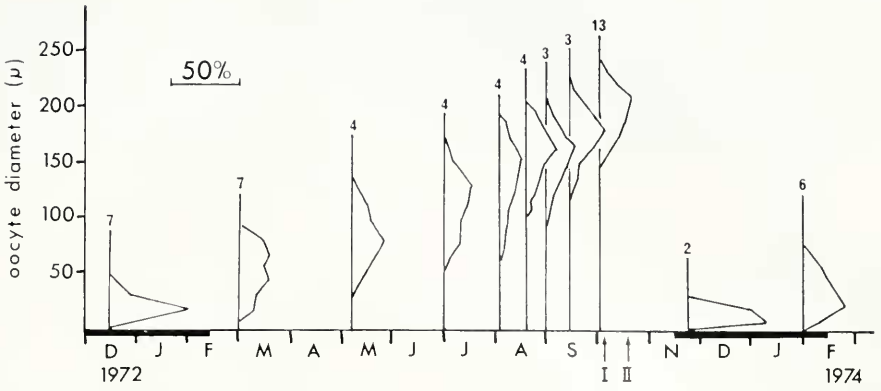

FIGURE 2. Fluctuation in the size-frequency structure of post-pachytene oocytes of *C. japonica*. Each of the eleven average frequency polygons was constructed from cytometric data from the number of females shown above the polygon. The thickened segments of the horizontal axis indicate the presence of oogonia and spireme oocytes. Arrows I and II mark, respectively, the spawnings of the first and third weeks of October 1973.

Within each ovary, the entire population of post-pachytene oocytes grew as a single generation (also called a cohort of oocytes). The growth of all oocytes as a single generation is rarely encountered in echinoderms, having previously been reported only for the asteroid *Patiriella regularis* (Crump, 1971) and possibly also for the asteroid *Asterias rubens* (Schlieper, 1957). This rare pattern of oocyte growth is certainly not a characteristic of crinoids in general, since the ovaries of most species contain more than one oocyte generation (Holland, unpublished observation).

In any female of *C. japonica* collected from late winter through early fall, the diameters of the largest and smallest oocytes differed by at least 80 μ. This wide range in oocyte diameters within each oocyte population was not an artifact caused by our sampling method, since similar ranges were found in random samples of unsectioned oocytes teased from fixed ovaries. Presumably, in each oocyte population, the largest cells were the first produced and thus the oldest, while the smallest cells were the last produced and thus the youngest; the following analysis of growth rates depends on the acceptance of the foregoing presumption.

Instantaneous relative growth rates of post-pachytene oocytes

The growth of two oocytes is shown in Figure 3. The curve at the left is for the largest (and presumably the first-produced) oocyte, and the curve at the right is for the smallest (and presumably the last-produced) oocyte. These cells, if spawned on 2 October, would be 321 and 229 days old respectively. The instantaneous relative growth rates (k) of the largest oocyte are as follows: 0.284 between 15 November and 24 November; 0.126 between 24 November and 14 December; 0.031 between 14 December and 1 March; and 0.012 between 1 March and 2 October. Similarly, instantaneous relative growth rates of the smallest

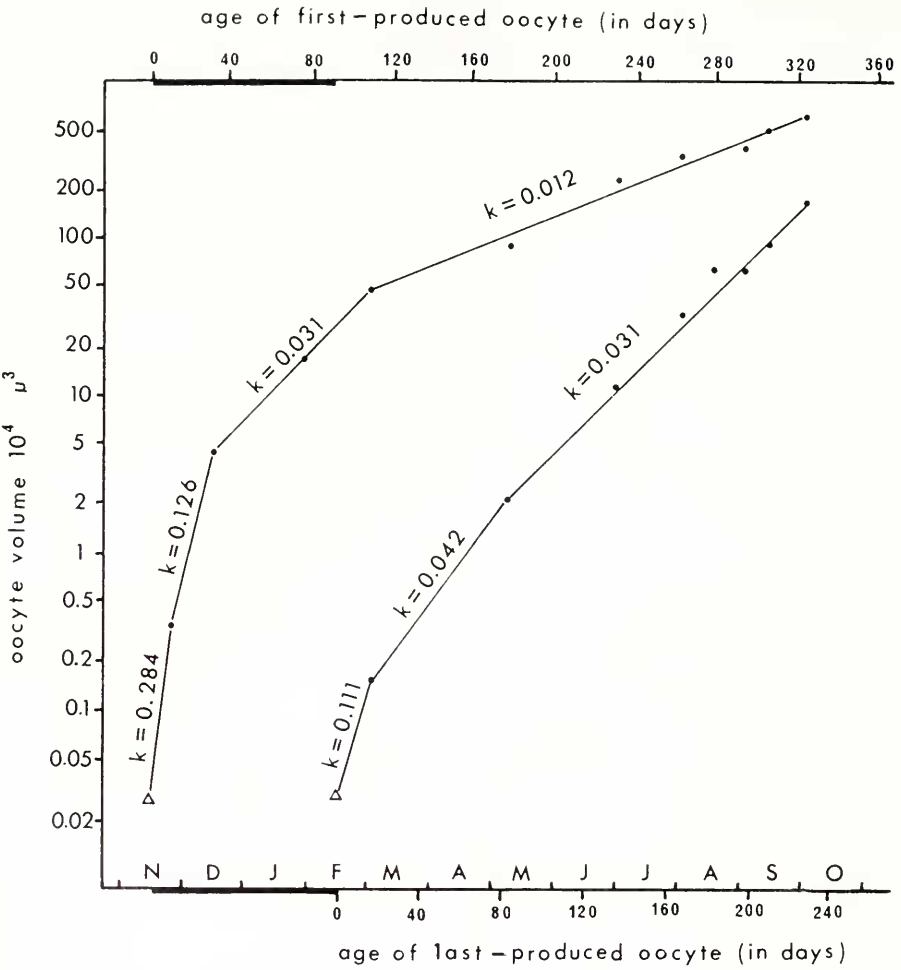

FIGURE 3. Instantaneous relative growth rates (k) of post-pachytene oocytes of *C. japonica*. The growth curve at the left is for the largest (and presumably the first-produced) oocyte, and the growth curve at the right is for the smallest (and presumably last-produced) oocyte. The triangular data point on each curve is the presumed volume on the birth date of the oocyte. The thickened segment of each horizontal axis indicates the presence of oogonia and spireme oocytes.

oocyte are as follows: 0.111 between 15 February and 1 March; 0.042 between 1 March and 7 May; and 0.031 between 7 May and 2 October.

The two curves of Figure 3 are not congruent. The last-produced oocyte, in comparison to the first-produced, has a slower early growth but a faster late growth. The reasons for these differences are not known. The tendency of the last-produced oocytes to catch up with the first-produced one during later growth is not great enough to prevent the wide range in oocyte diameters observed on the day of spawning. If one could plot growth curves for oocytes produced be-

tween mid-November and mid-February, one would no doubt obtain a family of curves falling between the two extremes plotted in Figure 3. All oocytes of *C. japonica*, no matter when they are produced, grow rapidly at first and then more slowly later. This pattern of oocyte growth contrasts markedly with that in *Strongylocentrotus purpuratus*, an echinoid in which the instantaneous growth rate is highest for medium-sized oocytes and not for small ones (Gonor, 1973b).

Changes in the total number of post-pachytene oocytes per ovary

The decline in the total number of post-pachytene oocytes per ovary is shown in Figure 4. Calculated numbers of oocytes per ovary were averaged (filled circles) and plotted against the date of collection. The eight collections represented were made from 1 March 1973 through 2 October 1973. In this seven-month period, during which no new cells were entering the cell population, the calculated number of oocytes per ovary declined from 3,360 to 1,348. By contrast, direct counts (triangles) revealed that, during the same period, the number of oocytes per ovary declined from 2,551 to 941. Evidently, the calculated values are somewhat too large, chiefly because the distal tapering of the ovary was not taken into account when the volume of the oocyte population was determined. In any case, it is safe to say that at least two to three times as many post-pachytene oocytes were originally produced as were present on the day of spawning. The decline in the number of oocytes was reflected by the presence of a few degenerating oocytes (and debris probably derived from them) in many of the ovaries sampled from March through October. It is not known if degeneration occurs at random in the oocyte population, irrespective of cell size. If one assumes that a large (40-armed)

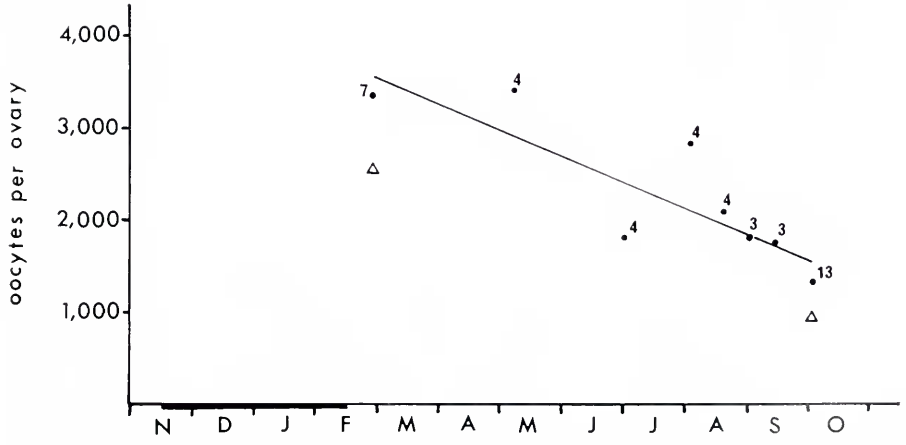

FIGURE 4. The decline in the total number of post-pachytene oocytes of *C. japonica* collected from early March through early October. The number of females averaged for each calculated number (filled circle) is shown above the date of collection; the regression line for these points was determined according to Sokal and Rholf (1969, chapter 14). The triangles show the number of oocytes determined by direct counts. The thickened segment of the horizontal axis indicates the presence of oogonia and spireme oocytes.

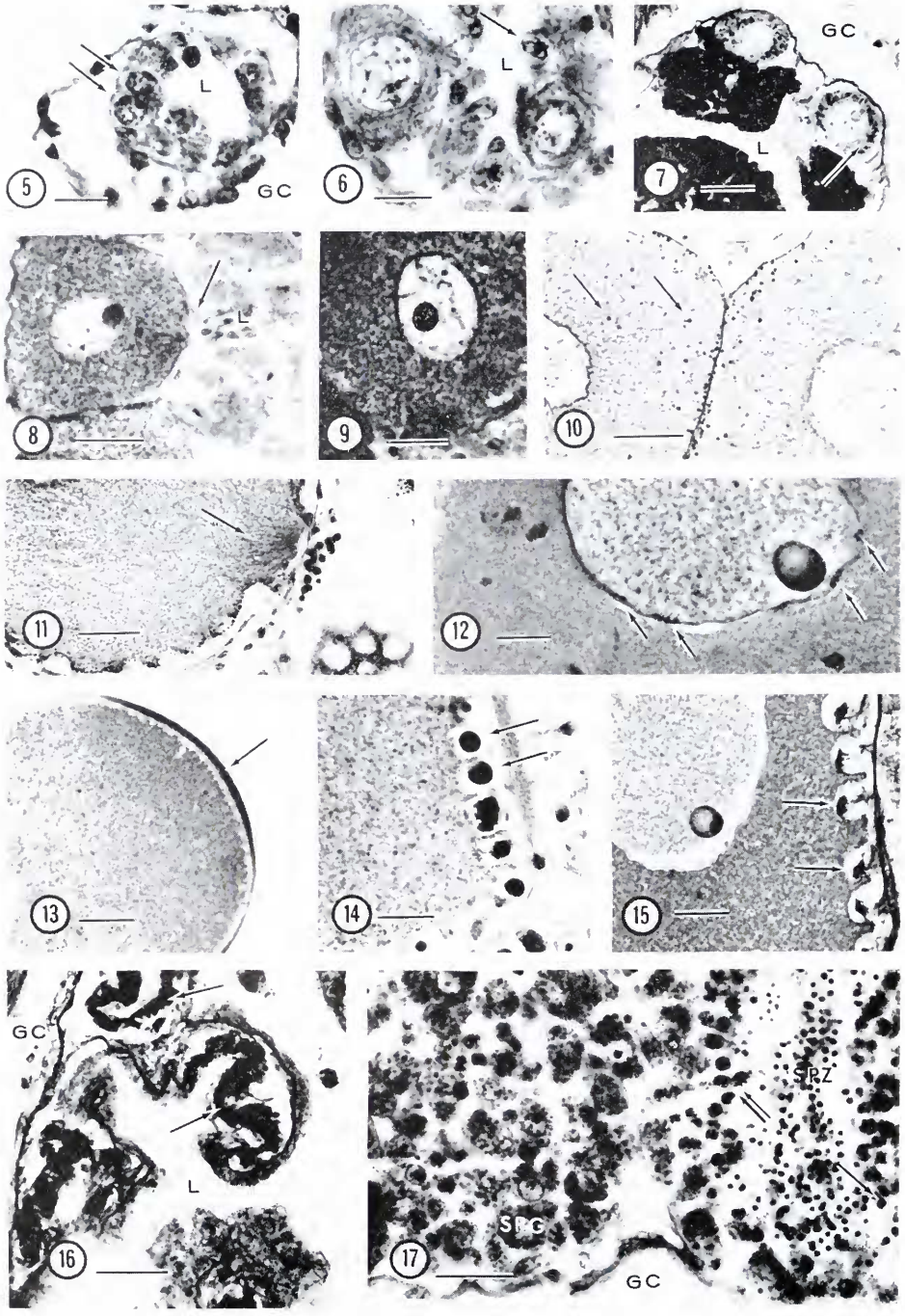

FIGURE 5. Cross section of a gonad of an unsexable animal collected 8 November 1973. Two gonial cells (arrows) are located in the inner layer of the gonad, which surrounds the

female has 2,000 ovaries which emit 1000 ova each, then a single female may spawn as many as two million ova.

The cytology of the cells of the inner layer of the unsexable gonad

The gonad, after spawning, passes through a brief phagocytic stage (which we will treat after the cells of the ripe gonads have been described) and enters a stage during which the sex of the animals cannot be established with certainty. During this unsexable stage, which lasts several weeks, a sex reversal by individual crinoids remains a possibility and should be investigated further. The inner layer of an unsexable gonad, like the one of 8 November (Fig. 5), consists of a simple epithelium of cuboidal cells. Some of these cells are about 8 μ across and contain

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gonadal lumen (L). The intermediate layer of the gonad appears empty of cells, while the outer layer of the gonad is conspicuous. The gonad is surrounded by the genital coelom (GC). The scale line is 10 μ; haematoxylin and eosin.

FIGURE 6. Cross section of an ovary of a female collected 14 December 1972. A spireme oocyte (arrow) is located in the inner layer of the ovary, which surrounds the ovarian lumen (L). Several post-pachytene oocytes, like the one at the upper left, bulge away from the lumen. The genital coelom is labeled GC. The scale line is 10 μ; haematoxylin and eosin.

FIGURE 7. Cross section of an ovary of a female collected 14 December 1972. Yolk granules (arrow) are present in the cytoplasm of post-pachytene oocytes. The lumen and genital coelom are labeled L and GC, respectively. Darkly stained accessory cells are present in the intermediate layer of the ovary. The scale in line is 20 μ; PAS.

FIGURE 8. The periphery of an ovary from a female collected 1 March 1973. The arrow indicates the association zone between an oocyte and non-germinal cells of the inner layer of the ovary. Toward the right of the figure are several accessory cells and a portion of the narrow ovarian lumen (L). The scale line is 25 μ; haematoxylin and eosin.

FIGURE 9. The periphery of an ovary from a female collected 1 March 1973. Diplotene chromosomes are conspicuous in the nucleus of the oocyte. The scale line is 20 μ; haemotoxylin and eosin.

FIGURE 10. Oocytes of a female collected 4 August 1973. Cortical granules (arrows) are scattered in the cytoplasm and are also abundant just beneath the plasma membrane. The scale line is 30 μ; azure A.

FIGURE 11. Dented oocytes of a female collected 24 September 1973. Cytoplasmic fibers (arrow) radiate from the region where a dented oocyte is associated with non-germinal cells of the inner layer of the ovary. A dented oocyte in tangential section is at the bottom right. The scale line is 25 μ; azure A.

FIGURE 12. A dented oocyte of a female collected 2 October 1973. Diakinesis chromosomes (arrows) lie at the periphery of the nucleus. The dark patches in the cytoplasm are annulate lamellae. The scale line is 25 μ; haematoxylin and eosin.

FIGURE 13. An oocyte of a female collected 24 September 1973. The extracellular jelly is in the form of a continuous layer (arrow). The scale line is 25 μ; PAS.

FIGURE 14. Dented oocytes of a female collected 27 September 1973. The extracellular jelly is mostly in the form of jelly spheres (arrows). The scale line is 25 μ; alcian blue and PAS.

FIGURE 15. A dented oocyte of a female collected 2 October 1973. The extracellular jelly is in the form of jelly clumps (arrows). The scale line is 25 μ; alcian blue.

FIGURE 16. The periphery of an ovary from a spawned female collected 7 October 1973. The ovarian lumen is labeled L, and the genital coelom is labeled GC. The chorions (arrows), which formerly surrounded the oocytes, have been left behind at ovulation in the inner layer of the ovary. The scale line is 30 μ; alcian blue and PAS.

FIGURE 17. The periphery of a testis from a male collected on 4 August 1973. Most of the cells at the left are spermatogonia (SPG). At the right are several spermatocytes (twin arrow), a spermatid (single arrow) and numerous spermatozoa (SPZ). The genital coelom is labeled GC. The scale line is 15 μ; haematoxylin and PAS.

a spherical nucleus about 6 μ in diameter. The nucleus contains one or two nucleoli about 1.5 μ in diameter and is surrounded by a thin shell of clear cytoplasm. It is reasonable to assume that these nucleolate cells are gonial cells. In addition to the gonial cells, the inner layer includes non-germinal cells characterized by a small amount of cytoplasm surrounding an oval nucleus (about 5 $\mu \times 3\ \mu$) that lacks a conspicuous nucleolus.

The cytology of the inner layer of the ovary until late September

By late November, ovaries are unequivocally recognizable, since their inner layers by then contain definitive female germinal cells. From late November through early February, the inner layer is made up of non-germinal cells, oogonia, spireme oocytes and post-pachytene oocytes. The oogonia, which resemble the gonial cells described in the preceding paragraph, differentiate into spireme oocytes (a term which covers the leptotene through pachytene stages of the first meiotic prophase). Each spireme oocyte is about 7 μ in diameter and has an anucleolate nucleus containing a tangle of threadlike chromatin (Fig. 6, arrow). Spireme oocytes give rise to post-pachytene oocytes (a term which covers the diplotene, diffuse, and diakinesis stages of the first meiotic prophase). Post-pachytene oocytes are about 8 μ in diameter when newly produced, but most are much larger. In the nucleus, one conspicuous nucleolus is invariably present. As post-pachytene oocytes enlarge, they soon bulge outward, away from the ovarian lumen (Figs. 6 and 7). From late February through spawning in October, the inner layer of the ovary consists only of non-germinal cells and post-pachytene oocytes.

Although the post-pachytene oocytes bulge away from the ovarian lumen, the inner edge of each oocyte remains in intimate association with the non-germinal cells of the inner layer (Figs. 8 and 11). Thus oocytes always belong to the inner layer of the ovary in *C. japonica*. In other crinoid species, oocytes have often been considered part of the intermediate layer of the ovary; this may well be an error of omission caused by the failure of previous workers (including Holland, 1971) to recognize the inconspicuous zone of cell-to-cell association. This zone was seen by Cuénot (1891) and by Dan and Dan (1941a), who referred to it as the oocyte stalk; however, those authors did not demonstrate that the base of the "stalk" was associated with non-germinal cells of the *inner* layer of the ovary. There is no light microscopic evidence that the association zone is the site of a massive transfer of nutrients from non-germinal cells to the oocyte.

From the birth date of a post-pachytene oocyte (which may be any time between 15 November and 15 February) until late September, cell structure depends on cell size. In Figure 18A (filled circles), the nuclear diameter has been plotted against the diameter of oocytes sampled prior to late September. The curve, by inspection, has an inflection at an oocyte diameter of 75 μ. The points to the left and right of the inflection are respectively fitted by the following regression lines: $y = 0.343x + 5.07$ and $y = 0.222x + 14.75$. Post-pachytene oocytes less than about 100 μ in diameter are in the diplotene stage, and their nucleoplasm contains strands of basophilic material (Figs. 6 and 9) that are presumably chromosomes. Oocytes larger than about 100 μ in diameter are in the diffuse stage (usage of Rhoades, 1961), and the nucleoplasm consists only of diffuse, flocculent material (Fig. 10).

The nucleolus of each oocyte is eccentrically located in the nucleus. In Figure 18B (filled circles) the nucleolar diameters have been plotted against the diameters of oocytes sampled prior to late September. The curve, by inspection, has an inflection at an oocyte diameter of 50 μ. The points to the left and right of the inflection are respectively fitted by the following regression lines: $y = 0.117x + 0.84$ and $y = 0.038x + 4.09$. Until late September, the nucleolus always consists of a spherical core surrounded concentrically by a thin cortex. Both core and cortex are strongly basophilic, the latter slightly more so than the former. Nucleolar staining with mercuric bromphenol blue varies in intensity from oocyte to oocyte; however, in general, the core stains more intensely than the cortex. In oocytes greater than about 75 μ in diameter, alcian blue stains the nucleolar core but not the cortex; thus, surprisingly, the core apparently contains nonsulfated acid mucopolysaccharide. No part of the nucleolus is ever stained by PAS.

Eosinophilic yolk granules begin to appear when post-pachytene oocytes reach a diameter of about 12 μ; soon thereafter, the yolk granules become ubiquitously and abundantly distributed throughout the oocyte cytoplasm. Each granule varies from about 0.5 μ to 2.5 μ in diameter. Cytochemically, the yolk granules stain intensely with PAS (Fig. 7) and moderately with mercuric bromphenol blue; these reactions indicate an abundance of neutral mucopolysaccharide and protein respectively. Since alcian blue and azure A do not stain the granules, little or no acid mucopolysaccharide is present. Vitellogenesis in *C. japonica* is very precocious in comparison to that in most other animals. Such precocious vitellogenesis is not characteristic of other crinoids that have been studied; in these, the first part of oocyte growth includes a long period of basophilic cytoplasm in which yolk granules are rare or absent (Chubb, 1906; Vannini, 1953; Urbani, 1955; Davenport and Davenport, 1966; Holland, 1971).

Cortical granules begin to appear in the cytoplasm of oocytes larger than 115 μ in diameter, excepting a few such cells in the sample of 7 May 1973. The cortical granules, which range in diameter from 1 μ to 3 μ, are stained dark blue (orthochromasia) with azure A (Fig. 10). Mercuric bromphenol blue stains the cortical granules only slightly darker than the yolk granules. Neither alcian blue nor PAS stain the cortical granules at all. In the collections of July, August and the first part of September, some cortical granules were scattered widely throughout the oocyte cytoplasm (Fig. 10, arrows), while the rest of the cortical granules were located just beneath the plasma membrane. There is little doubt that the cortical granules scattered in the cytoplasm are being produced there; then, after having been produced, they migrate to the extreme periphery of the oocyte where they accumulate.

The inner edge of each post-pachytene oocyte, as already described, remains in intimate association with non-germinal cells of the inner layer of the ovary. The oocyte cytoplasm, just subjacent to this association zone, often contains fibers which radiate toward the center of the cell. These fibers (some of which are visible, but not conspicuous in Fig. 8) stain darkly with haematoxylin and also with azure A (showing orthochromasia). Although the function of these fibers is not known, they are evidence of an early cytoplasmic polarity in the post-pachytene oocytes.

The oocyte surface appears smooth by light microscopy in collections made be-

fore late September. Around oocytes that have reached about 75 μ in diameter, an extracellular coat, which we will call the chorion, becomes faintly stainable with alcian blue. This chorion becomes more conspicuous around larger oocytes, but never exceeds half a micron in thickness; it continues to stain with alcian blue and also stains weakly with PAS, mercuric bromphenol blue and azure A (showing beta metachromasia). These cytochemical reactions indicate that the chorion includes some protein and acid mucopolysaccharide. Even when well-developed, the chorion never intervenes between the oocyte and the adjacent non-germinal cells of the inner layer; instead, each chorion is probably continuous with the basal lamina underlying the non-germinal cells of the inner layer (this relationship will be discussed further below).

The cytology of the inner layer of the ovary from late September to spawning

Between late September and spawning in October, the inner layer of the ovary still consists only of non-germinal cells and post-pachytene oocytes. By this time, the nuclei of the several dozen non-germinal cells adjacent to each oocyte become more spherical and more basophilic than before (Fig. 11). Oocyte structure no longer depends on cell size; instead, all the oocytes in a given ovary begin to differentiate almost simultaneously, even though the diameters of the largest and smallest cells in the population differ by about 80 μ. During the final weeks of oogenesis, conspicuous morphological changes occur both in the nucleus and in the cytoplasm.

A disproportional swelling of the oocyte nucleus begins after mid September (Fig. 18A). The swelling is significant, since the confidence limits of the following regression lines do not overlap: $y = 0.222x + 14.75$ (15 September); $y = 0.212x + 30.65$ (24 September); and $y = 0.128x + 63.19$ (2 October). Moreover, in the collections from 27 September through 2 October, chromosomes reappear in most of the oocytes (Fig. 12, arrows). Such oocytes have entered the diakinesis stage of the first meiotic prophase. The diakinesis chromosomes are short, basophilic structures located at the periphery of the nucleus; one of them is typically associated with the nucleolus. Elsewhere in the nucleus, the nucleoplasm consists of flocculent material.

The oocyte nucleolus also begins disproportional swelling after mid September (Fig. 18B). The swelling is significant, since the confidence limits of the following regression lines do not overlap: $y = 0.038x + 4.09$ (15 September); $y = 0.002x + 11.85$ (24 September); and $y = 0.018x + 13.31$ (2 October). By 2 October, the shape of each nucleolus has become slightly ovoid (Fig. 12), but not as conspicously so as some of the nucleoli in the figures of Dan and Dan (1941a). Structurally, the nucleolus no longer consists of a core surrounded concentrically by a thin cortex. Instead, the former core, identifiable by its affinity for alcian blue (Fig. 15), is now located at one side of the nucleolus; the core material remains basophilic and still stains with mercuric bromphenol blue. The remainder of the nucleolus probably corresponds to the former cortex; however, this presumed cortical material, in comparison to the former cortex, is much less basophilic, but stains more intensely with mercuric bromphenol blue. Some, but not all, of the nucleoli at this stage contain one or several nucleolar vacuoles (Fig. 15).

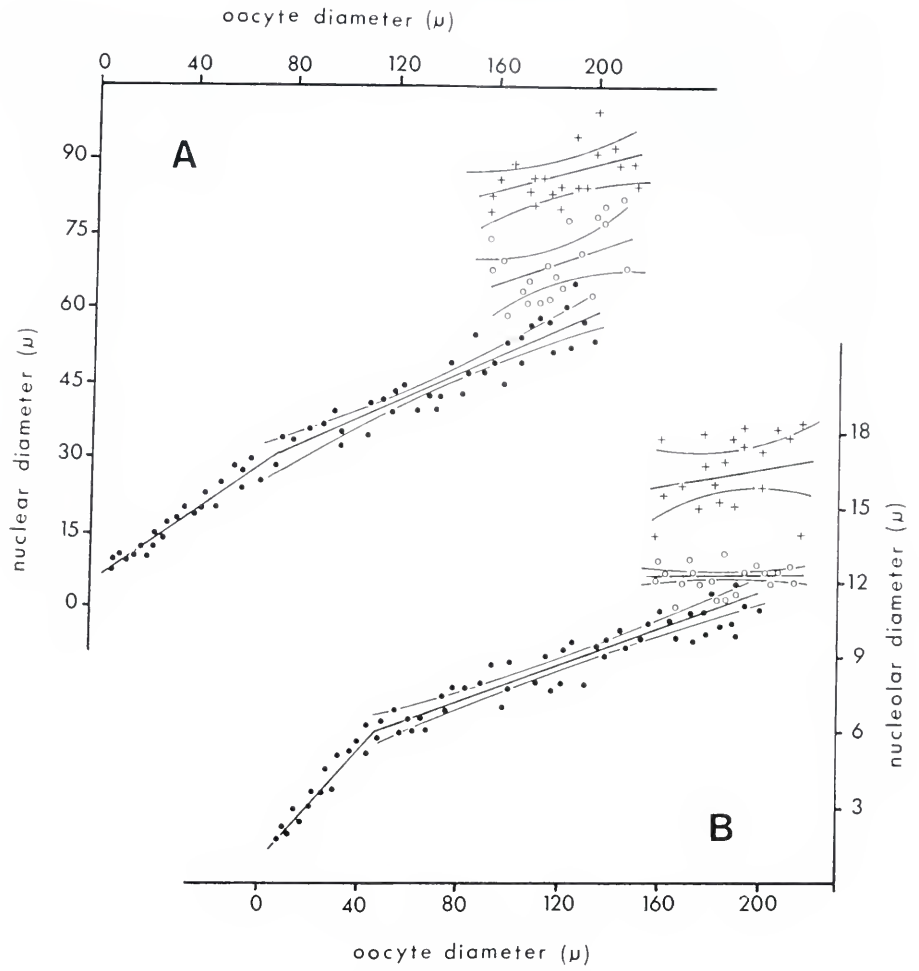
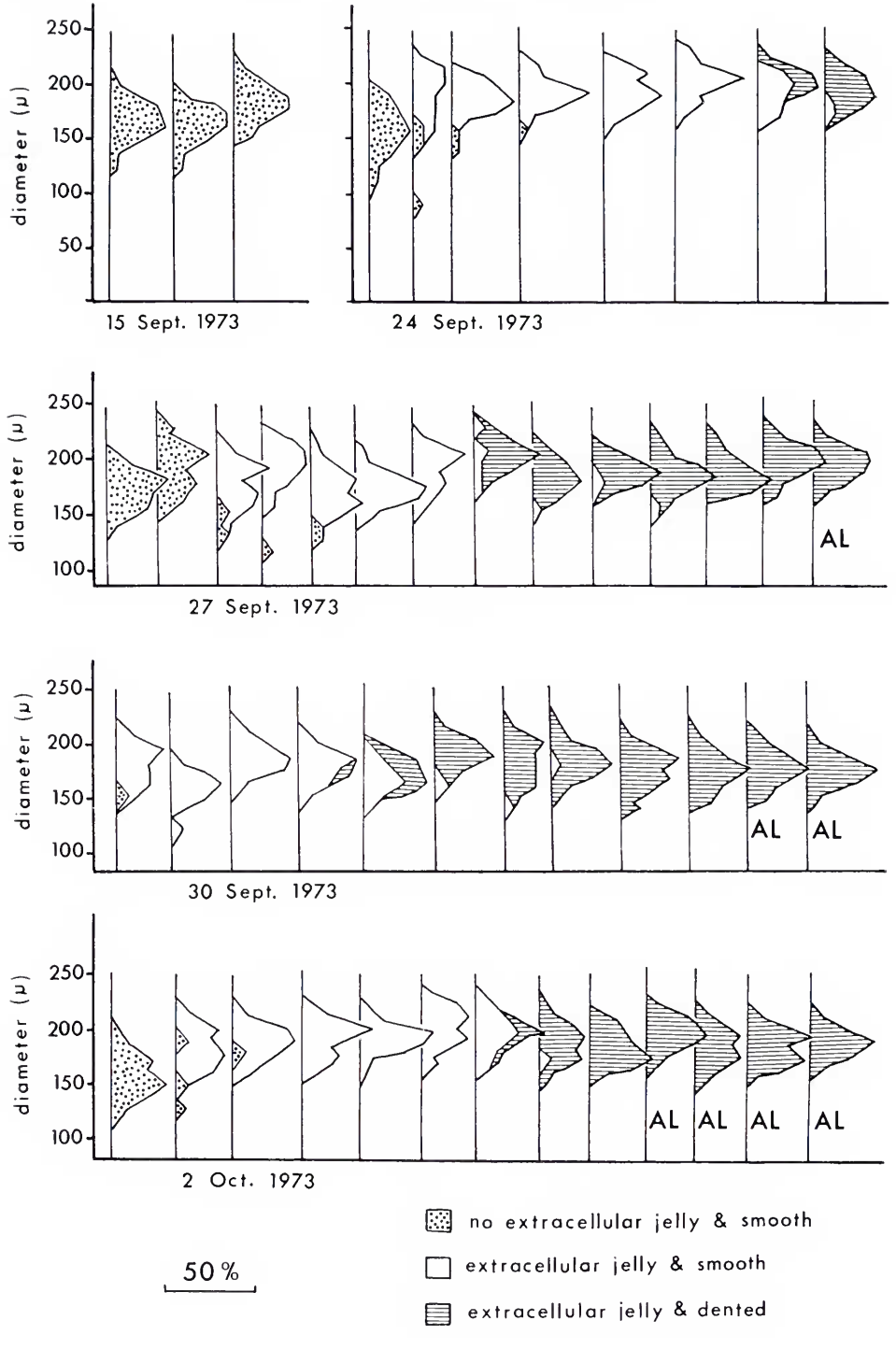


FIGURE 18. (A) The growth of the nucleus relative to the size of the post-pachytene oocyte; (B) the growth of the nucleolus relative to the size of the post-pachytene oocyte. In both graphs, the filled circles are diameters measured from specimens collected from 14 December 1972 through 15 September 1973; the open circles are diameters measured from specimens collected on 24 September 1973; and the crosses are diameters measured from specimens collected on 2 October 1973. The regression lines and 95% confidence limits have been calculated according to Sokal and Rholf (1965, chapter 14).

The cortical granules, from 15 September through 2 October, are located just beneath the plasma membrane of the oocyte (Fig. 11). Their absence from deeper regions of the cytoplasm presumably reflects the cessation of cortical granule production by mid September.

Annulate lamellae were present in the oocyte cytoplasm of one female collected on 27 September. By light microscopy, annulate lamellae (which were identified as such by our unpublished electron microscopy) appear in the cytoplasm as baso-


diameter (μ)
250
200
150
100
50
15 Sept. 1973
24 Sept. 1973
27 Sept. 1973
AL
30 Sept. 1973
AL
AL
2 Oct. 1973
AL
AL
AL
AL
50 %
no extracellular jelly & smooth
extracellular jelly & smooth
extracellular jelly & dented

philic patches ranging from about 3 μ to 15 μ in average diameter (Fig. 12). In oocytes of one female of 27 September, some annulate lamellae were closely associated with the edge of the nuclear envelope, possibly being elaborated there. In the collections of 30 September and 2 October, annulate lamellae were present in the oocytes of two and four females respectively (Fig. 19).

Extracellular jelly appeared around the oocytes of most females in the population during the third week of September 1973 (Fig. 19), apparently regardless of whether the females were destined to spawn in the first or the third week of October. Within a given ovary, all the oocytes became surrounded by jelly almost simultaneously in the context of the whole course of oogenesis; actually, the smallest oocytes tended to become surrounded by jelly a few hours or days after the larger oocytes.

The extracellular jelly, when it first appeared, was a layer about 7 μ thick between the plasma membrane and the chorion. This layer (Fig. 13, arrow) was an unbroken sheet of homogeneous material, often limited to the oocyte hemisphere nearest the ovarian lumen. This jelly stained intensely with PAS, but remained unstained by azure A and alcian blue; thus neutral mucopolysaccharide was present, but acid mucopolysaccharide was undetectable at this stage. A strong reaction with mercuric bromphenol blue indicated that protein was also present. The origin of the extracellular jelly is not known with certainty; however, it seems likely that most or all of the jelly is produced intracellularly and secreted by the oocyte itself.

The formerly smooth oocyte surface became dented (Fig. 19) a few days after the advent of the extracellular jelly, at least in those females destined to spawn in the first week of October. Within a given ovary, all the oocytes became dented within a relatively short time of one another (perhaps within hours or, at most, a few days). Denting began with the appearance of hundreds of pits in the oocyte surface (Fig. 11). The diameter of each pit was roughly 15 μ to 20 μ and the depth was about 10 μ (Figs. 14 and 15). Such pits were never widely scattered over the oocyte surface; instead they were almost contiguous, as can be seen from the tangentially sectioned oocyte at the bottom right in Fig. 11. In some oocytes of 24 September, the pits were relatively shallow and had presumably just been formed. Occasionally, an oocyte was seen with one smooth hemisphere and one shallowly pitted hemisphere; this pattern indicates that denting might start at one point on the surface and spread from there. The cytoplasmic fibers (Fig. 11, arrow), which are present as denting begins, vanish shortly thereafter; it is not known if they play a role in surface denting.

For a given oocyte, the advent of the dented surface was closely correlated with the breaking up of the extracellular jelly into numerous isolated spheres, each about 10 μ in diameter (Fig. 14). The histochemistry of most of these spheres resembled that of the jelly layer of smooth oocytes; neutral mucopolysaccharide and protein were conspicuous components, but no acid mucopolysaccharide was detectable. It is not known with certainty whether the jelly spheres caused denting or the denting caused jelly spheres. To picture the first possibility,

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FIGURE 19. The size-frequency structure of the post-pachytene oocytes of each female collected from 15 September 1973 through 2 October 1973; frequency polygons of females having annulate lamellae in their oocytes have been labeled AL.

one could imagine that the jelly layer surrounding a smooth oocyte spontaneously broke into numerous jelly spheres, and that the oocyte cytoplasm then intruded into the spaces between them. To picture the second possibility, one could imagine that the jelly layer surrounding a smooth oocyte was divided into numerous jelly spheres by cytoplasmic ridges actively elevating from the oocyte surface. The second possibility, that denting causes jelly spheres, is the more likely, since a dented area of oocyte surface may occasionally be overlain by an unbroken jelly layer. One additional mode of jelly sphere formation might be the secretion of jelly into a pre-existing pit after denting has been completed.

The jelly spheres, soon after being formed, undergo further morphological and histochemical transformation. Each sphere, which consists of relatively homogeneous material (Fig. 14, arrows) appears to be converted into a condensed clump of relatively fibrous material (Fig. 15, arrows). These fibrous jelly clumps stain intensely with alcian blue, but do not stain with PAS or azure A; such histochemical properties define a nonsulfated acid mucopolysaccharide (Spicer, 1963). Mercuric bromphenol blue fails to stain the jelly clumps, indicating that protein is absent or masked from reacting with the stain. A given pit may sometimes contain both clump and sphere material; in such cases, the clump material is always located nearest the bottom of the pit. Each jelly clump is probably produced by an extracellular conversion of the jelly sphere's neutral mucopolysaccharide to nonsulfated acid mucopolysaccharide, perhaps under the influence of enzymes released from the oocyte.

From Figure 19, it is reasonable to assume that most of the females which failed to spawn on 2, 3 and 4 October had smooth oocytes with extracellular jelly. Such oocytes were still present in the eight unspawned females collected on 7 October. Of seven unspawned females collected on 11 October, four had smooth oocytes with extracellular jelly, and three had dented oocytes with extracellular jelly. Thus the oocytes of the unspawned females were apparently becoming dented about a week before the spawnings of 17 and 18 October. It is probable, therefore, that oocytes become dented a week before spawning, regardless of whether spawning took place in the first or in the third week of October. Other oocyte development dependent on the time before spawning might be the production of annulate lamellae and the appearance of diakinesis chromosomes (unfortunately, these phenomena were not studied just prior to the spawnings of the third week of October).

In the hours leading up to spawning, ovulation occurs, and the oocytes mature into ova; these phenomena will only be summarized here, since they are more appropriately demonstrated by electron microscopy (Holland, unpublished observations) than by light microscopy. At ovulation, the oocytes squeeze between the non-germinal cells of the inner layer of the ovary and enter the lumen. In the ovarian lumen, the oocytes quickly pass through the two meiotic divisions and become ova. Soon thereafter, the ova are expelled from the lumen through a simultaneous rupture of the gonadal wall and the lateral body wall of the pinnule.

The cytology of the inner layer of the ovary soon after spawning; the phagocytic stage

At ovulation, as the oocytes pass from the inner layer to the lumen, the chorions of the oocytes are left behind, much collapsed and thickened, as a part of the inner

layer of the ovary (Fig. 16, arrows). The collapsed chorions are histochemically the same as the ones that surrounded the unovulated oocytes. In the days after spawning, one can see that the collapsed chorions are continuous with a basal lamina underlying the non-germinal cells of the inner layer of the ovary. Presumably, this basal lamina is always present, but is too thin during much of the year to be demonstrable by light microscopy. Collapsed chorions can be recognized in the ovaries for several weeks after spawning, but then disappear, probably by destruction *in situ* by non-germinal cells of the inner layer or intermediate layer.

Most spawned ovaries retain about a dozen oocytes and ova; the unspawned oocytes remain a part of the inner layer, and the unspawned ova remain free in the ovarian lumen. Within a few weeks after spawning, all unspawned germinal cells have broken down, and their remains have been taken up phagocytically by the non-germinal cells of the inner layer. During this phagocytic stage, these non-germinal cells range in shape from cuboidal to low columnar.

The cytology of the inner layer of the testis until spawning

The testes, after spawning, pass through a phagocytic stage (described below) and an unsexable stage (which has already been described) . By mid December, the unsexable stage ends, and the testes become unequivocally recognizable, since definitive spermatogonia are present along with non-germinal cells in the inner layer. Definitive speratogonia, while generally resembling gonial cells of unsexable animals, are characterized by the presence of small cytoplasmic granules. Such granules, which range in diameter from about 0.5 μ to 1.5 μ, stain moderately with PAS, weakly with mercuric bromphenol blue and not at all with azure A or alcian blue. Comparable granules have never been reported in the spermatogonia of other echinoderms.

Throughout the winter, spring and early summer, the spermatogonia divide mitotically without differentiating into more advanced germinal cells. During this part of the annual reproductive cycle, as already mentioned, the maximum mean cell cycle time of the spermatogonia is 35 days. As a result of this cell division, the inner layer of the testis becomes about eight spermatogonia thick by 1 July. A few of the spermatogonia are about twice the size of the others; these larger cells might be about to enter mitosis. The testis of *C. japonica* might contain both multiplying spermatogonia and terminal spermatogonia (in the terminology of Giese and Pearse, 1974) ; however, these two cell varieties are indistinguishable by morphological criteria. Some non-germinal cells probably continue to be present in the inner layer, but we could not find them among the abundant spermatogonia.

By the collection of 4 August, the spermatogonia have begun to differentiate into more advanced germinal cell types (Fig. 17). The inner layer of the testis now includes spermatogonia, spermatocytes (presumably both primary and secondary), and spermatids, while the testicular lumen contains some spermatozoa. Thus, the cytodifferentiative sequence from spermatogonium to spermatozoa is completed in a month at the most. In the testis of *C. japonica*, the spermatocytes occupy the more luminal parts of the inner layer and appear as densely basophilic nuclei about 4 μ in diameter surrounded by clear cytoplasm (Fig. 17, twin arrow). The spermatocytes are often seen in meiotic divisions. The spermatids, which occur right next to the lumen, appear as dense nuclei about 2.5 μ in diameter

(Fig. 17, single arrow). In the testicular lumen, the heads of the spermatozoa are dense, basophilic spheres just under 2 μ in diameter.

Spermatogonial differentiation continues throughout August, September and early October; thus, at spawning, the first-produced spermatozoon is roughly ten weeks older than the last-produced spermatozoon. Shortly before spawning in October, the inner layer of the testis consists mainly of spermatocytes and spermatids, and spermatogonia are much less abundant than earlier in the summer. Apparently, the rate of spermatogonial differentiation has become far greater than the rate of spermatogonial division, and the cell population has, therefore, been depleted.

The cytology of the inner layer of the testis soon after spawning; the phagocytic stage

When males spawn, the cells expelled from the testis apparently include not only spermatozoa, but also many of the less advanced germinal cells of the inner layer (Fig. 1B). After spawning, the testis invariably retains some spermatozoa in the lumen. During the next few weeks, non-germinal cells become conspicuous in the inner layer; they appear to engulf spermatozoa from the lumen and probably also engulf the less advanced germinal cells remaining in the inner layer. After a few weeks of phagocytosis, no germinal cells of any kind, not even spermatogonia, can be found in the testis.

Discussion

In November, gonial cells first appear in the gonads of *Comanthus japonica*. This event, which establishes a rough gametogenic synchrony among the individual animals of the population, is probably initiated in both sexes by an environmental signal dependent on the time of year (perhaps shortening photoperiod or falling sea temperature). Unfortunately, it is not presently known whether the advent of the gonial cells is a matter of cell migration or cell differentiation. The gonial cells might migrate into the gonad from elsewhere in the animal (as proposed by Hamann, 1888, p. 83), or they might differentiate *in situ* from non-germinal cells of the inner layer of the gonad. In the testes, spermatogonia divide mitotically throughout the winter, spring and early summer without entering meiosis (*i.e.*, differentiating into spermatocytes). The deveolpmental block between spermatogonia and spermatocytes is not overcome until July, August and September. In the ovaries, by contrast, the oogonia undergo relatively little mitotic division and soon enter meiosis (*i.e.*, differentiate into oocytes). This differentiation continues through the winter until mid February, by which time the supply of oogonia apparently becomes exhausted. Because male and female germ cells do not follow parallel courses after the gonial cells have appeared, it is reasonable to assume that one set of controls operates in males and another operates in females during much of the year.

Oocyte cytology depends on cell size throughout the winter, spring and summer; moreover, there is neither a suspension of oocyte growth nor a sudden increase in the instaneous relative growth rate. These characteristics indicate that much of oocyte growth is autodifferentiative (in the sense of Charniaux-Cotton, 1973). Then, from late September until spawning in October, oocyte cytology

no longer depends on cell size; instead, all the oocytes in a given ovary begin to differentiate almost simultaneously, in spite of the 80 μ range in their diameters. Such a response pattern is a good indication that environmental signals are acting on the entire cell population via nervous and endocrine mechanisms. Unfortunately, nothing definite is presently known about reproductive endocrinology in crinoids. A study of the reproductive endocrinology of *C. japonica* would be of special interest because of the high degree of spawning synchrony in this species.

On the day of spawning each year, a large female specimen of *C. japonica* emits a total of about two million ova. In making this calculation, we ignored the few unspawned ovaries that can usually be found scattered at random among the spawned ovaries after the ova have been emitted (Dan and Dan, 1941b); we also ignored the dozen or so gametes remaining in each spawned ovary. In spite of these sources of error, the fecundity of a female can be determined for *C. japonica* far more accurately than for most other animals broadcasting large numbers of eggs into the sea water. Under good laboratory conditions, nearly all the spawned ova are fertilizable, in spite of the wide range in cell diameters. It is not presently known if the size of a zygote at the start of development ultimately influences the individual's chance of survival during the subsequent life history.

Within a few minutes after fertilization, the zygote of *C. japonica* becomes surrounded by an elaborate fertilization membrane, which has been described by Holland and Jespersen (1973). The pattern of ridges on the outer surface of the fertilization membrane is very reminiscent of the surface topography of the dented oocyte. There can be little doubt that the oocyte surface is a template that somehow imposes its form on the fertilization membrane. The details of how the surface pattern is transferred during fertilization and the cortical reaction have yet to be elucidated.

The present investigation was made possible by the cordial hospitality of Director Hideshi Kobayashi and the staff of the Misaki Marine Biological Station of the University of Tokyo. Special thanks are due to Mrs. Linda Holland for doing our statistics and to Dr. Shonan Amemiya, Dr. Bo Fernholm and Dr. Tomoyuki Ichikawa for helping us to collect and make field observations. Our paper was greatly improved by the critical readings of Professor R. B. Clark, Dr. P. J. W. Olive and Dr. J. S. Pearse.

Summary

1. Periodic sampling of a Japanese population of an unstalked crinoid, *Comanthus japonica*, demonstrated an annual reproductive cycle delimited by spawning in October.

2. In both sexes, the first weeks after spawning were a time of phagocytosis of unspawned germinal cells by non-germinal cells of the inner layer of the gonad.

3. In November, gonial cells made their appearance in the inner layer of the gonad, which was unsexable for several weeks.

4. In females, during the latter part of November, oogonia began differentiating into oocytes and continued to do so until mid February; this resulted in an

oocyte population in which the largest cells were three months older than the smallest cells.

5. During the winter, spring and summer, the oocyte population grew as a single generation until spawning in October; although there was some tendency for the smaller oocytes to catch up with the larger ones, oocyte diameters ranged from about 145 μ to 225 μ on the day of spawning. The instantaneous relative growth rates of all oocytes were high at first and then decreased during later growth.

6. In each ovary, at least two to three times as many oocytes were initially produced as were finally present just before spawning; a large female emitted about two million gametes on the day of spawning each year.

7. Prior to late September, oocyte morphology depended on oocyte size. The following changes occurred at the following oocyte diameters; yolk granule synthesis started at 12 μ; the diplotene chromosomes disappeared at 100 μ; and cortical granule synthesis started at 115 μ.

8. From late September to spawning in October, all the oocytes in an ovary began to differentiate almost simultaneously, irrespective of their size. During this short period, the nucleus and nucleolus swelled conspicuously, extracellular jelly appeared, and the oocyte surface became dented with hundreds of pits, each about 10 μ deep. Finally, several days before spawning, annulate lamellae appeared in the cytoplasm, and diakinesis chromosomes appeared in the nucleus.

9. In males, the spermatogonia divided mitotically throughout the winter, spring and early summer without differentiating into more advanced germinal cell types; during this period, the maximum mean cell cycle time was about 35 days. Subsequently, from mid summer through spawning in October, the spermatogonia differentiated, via spermatocytes and spermatids, into spermatozoa.

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